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The Effects of Repeated Low-Level Sarin Exposure on Muscarinic M_1 Receptor Binding, Amyloid Precursor Protein Levels and Neuropathology

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14. ABSTRACT
The objective of this study was to determine whether there are significant changes in muscarinic M_1 receptors (m_1AChR), levels of amyloid precursor protein (APP), and neuropathology following repeated exposure to low levels of sarin. Guinea pigs were exposed 5 days/week/two weeks to either saline or one of two sarin doses (0.2 or 0.4 x LD_{50}; s.c.). Experimental parameters were assessed at five time points: exposure day 10 (E10) and at 3 (P3), 10 (P10), 30 (P30) and 100 (P100) days post-exposure. No difference was observed among groups in either receptor density (B_{max}) or receptor affinity (K_d) following cortical m_1AChR binding. Western blotting revealed increases in membrane-bound cortical APP levels in both sarin groups at P3, with an increase in only the 0.4 x LD_{50} group at P30. APP levels were equivalent across groups at P100. H&E staining revealed no brain lesions in either sarin group, and there were no cardiac abnormalities. MAP-2 staining was performed; no difference in staining intensity was observed in either sarin group. Current data suggests that the initial depression of acetylcholinesterase is not of sufficient magnitude and duration to result in persistent neurochemical or neuropathological changes, or in physiological, electroencephalographic, or behavioral alterations.

15. SUBJECT TERMS
Acetylcholine; Acetylcholinesterase; Amyloid precursor protein; Brain Regions; Cortex; MAP-2; Microtubule associated protein; Muscarinic; Nerve agents; Organophosphorus; Pirenzepine; Receptor Binding; Sarin

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ABSTRACT

The objective of this study was to determine whether there are significant changes in muscarinic M_1 receptors (m_1AChR), levels of amyloid precursor protein (APP), and neuropathology following repeated exposure to low levels of the organophosphorus nerve agent sarin (8.4 or 16.8 μg/kg, equivalent to 0.2 or 0.4 x LD_{50}; s.c.). Guinea pigs were exposed 5 days/week for two weeks (Mon-Fri) to either saline or one of the two sarin doses. To determine whether persistent or delayed effects were present, experimental parameters were assessed at five time points: the day of the tenth exposure (E10) and at 3 (P3), 10 (P10), 30 (P30) and 100 (P100) days post-exposure.

No difference was observed among groups in either B_{max} (receptor density) or K_d (receptor affinity) following cortical M_1 muscarinic receptor binding using [^{3}H]-Pirenzepine, across all five time points. Using a monoclonal antibody to APP, Western blotting revealed a transient increase in membrane-bound cortical APP levels in both sarin groups at P3, with an increase in only the 0.4 x LD_{50} group at P30. APP levels were equivalent across groups at P100. Hematoxylin and eosin (H&E) staining revealed no lesions in either of the sarin groups, and there were likewise no cardiac abnormalities observed. To assess for neuronal injury, microtubule associated protein (MAP-2) staining was performed; however, no difference in staining intensity was observed in any sarin-exposed group.

Although our previous data found that red blood cell (RBC) and regional brain acetylcholinesterase (AChE) activity were significantly depressed by sarin treatment following E10 with a steady increase back to baseline levels by P100, these current data suggest that the initial depression of AChE is not of sufficient magnitude and duration to result in persistent changes that cause neurochemical or neuropathological alterations. The data also support our previous findings of no persistent physical, electroencephalographic or behavioral alterations.

I. INTRODUCTION

Much remains to be learned about the effects of exposure to asymptomatic levels of the organophosphorus (OP) nerve agents. It has been reported that exposure to sublethal doses of the OP nerve agent soman in rats results in a reduction of cortical muscarinic receptors (mAChRs; Churchill et al., 1984-a, 1985). This decrease has been described as an adaptive down-regulation (Churchill et al., 1985) that is likely a response to the OP inhibition of acetylcholinesterase (AChE) and the subsequent accumulation of synaptic acetylcholine (ACh). Likewise, exposure to the potent cholinergic agonist BM 123 results in a reduction of mAChRs in rat cortex and striatum (Russell et al., 1986). Exposure to less potent OP agents, such as paraoxon (PX), disulfoton or diisopropyl fluorophosphate **(**DFP), has also resulted in a reduction of mAChRs in both rat brain tissue (Churchill et al., 1984-b; McDonald et al., 1988; Raffaele et al., 1990; Fitzgerald and Costa, 1992; Ehrich et al., 1994, Zheng et al., 2000) and SH-SY5Y cells, which have a high density of phosphoinositide hydrolysis-linked muscarinic receptors (Katz and Marquis, 1992). Depending on the ligand chosen, these studies have observed reductions in the general population of mAChRs as well as in several muscarinic subtypes, including M_1, M_2 and M_3 receptors. Additionally, one study in calf caudate membranes found that PX reduced the number of non-specific muscarinic binding sites (using quinuclidinyl benzilate; QNB) at PX concentrations that do not significantly inhibit AChE activity (Katz and Marquis, 1989). Khan et al. (2000) found time point-dependent biphasic changes in cortical nicotinic and M_2 muscarinic binding in rats at concentrations of sarin (0.01 and 0.1 x LD_{50}) that were below the doses used in the current study. McDonald et al. (1988) noted no changes in cortical receptor affinity following binding with QNB, while a 16-28% decrease in receptor density was observed.

The amyloid precursor protein (APP) plays a central role in the pathogenesis of Alzheimer's disease (AD) (see Roberson and Harrell, 1997, for review). A fragment of APP called β-amyloid is found in amyloid plaques, one of the hallmark neuropathological markers of AD. A number of neurotransmitter systems and receptors have been associated with the normal metabolism of APP, including the cholinergic and serotonergic systems (Nitsch et al., 1992, 1996; Lee et al., 1995; Muller et al., 1997). Any perturbation of those systems associated with normal APP metabolism, such as would be caused by the inhibition of AChE activity in the brain following OP exposure, could cause a change in APP processing. Since altered APP metabolism is thought to play a role in the neurodegenerative cascade seen in Alzheimer's disease, changes in either the metabolism or levels of APP following OP exposure could signal the onset of potentially deleterious processes in the brain. In one previous study, an increase in APP mRNA was observed following repeated low-level VX exposure (0.4 and 0.6 x LD_{50} VX; LD_{50} = 9 μg/kg) in mice (Blanton et al., 2004; see Figure 2/Cluster F: Down syndrome critical region gene).

It is well known that seizure-producing levels of the OP nerve agents produce severe brain and cardiac damage in rats (McDonough et al., 1995, 1998; For review see McDonough and Shih, 1997). The seizure process is initiated by changes in the cholinergic neurotransmitter system (the initial or "cholinergic phase" of the process); however, the neuronal excitation of the seizure per se perturbs other neurotransmitter systems (McDonough and Shih, 1997). As activity of the cholinergic system declines (called the "transition phase") the glutamate neurotransmitter system becomes dominant, signaling glutamatergic or "late phase" control over the seizure mechanisms (McDonough and Shih, 1997). M_1 receptors are the most prevalent pharmacological subtype in cortex and striatum (Cortes and Palascios, 1986; Levey et al., 1991) and the cholinergic system-mediated effects are likely predominantly provoked through the

effects of activation of m_1AChRs on phosphatidylinositol hydrolysis (Brown et al., 1984). Because of the extensive data available on OP seizure-elicited cardiac damage (McDonough et al., 1989; 1995) and also because of the location of cholinergic M_2 receptors in heart (Silviera et al., 1990), cardiac pathology was also evaluated in the current study.

Animals were exposed to saline or to one of two doses of sarin (0.2 or 0.4 x LD_{50} sarin; 8.4 or 16.8 μg/kg), five days/week (Mon-Fri) for two weeks. The doses were asymptomatic, although significant reductions in brain (0.4 x LD_{50}) and red blood cell (RBC) AChE (0.2 and 0.4 x LD_{50}) occurred following exposure 10 with a steady increase back to baseline levels by 100 days post-exposure (Roberson et al., 2004). The results reported here include those of cortical receptor binding assays using [^{3}H]-Pirenzepine (m_1AChR ligand; Hammer et al., 1980), Western blotting using an antibody to APP in cortex, and neuropathological analysis using hematoxylin and eosin (H&E) and microtubule-associated protein (MAP-2) staining.

II. METHODS

2.1 Animals

Male Hartley guinea pigs (Crl: (HA) BR; N = 150), obtained from Charles River Laboratories (Kingston, NY) with an average weight of 350 g at baseline and an average weight of 884 g at P100, were housed individually in hanging cages in a temperature (20± 2º C) and humidity (50± 10%) maintained room, with an alternating 12-hr light/dark cycle with lights on at 0600 hours. Standard laboratory chow and water were available *ad libitum*. The animal care program at USAMRICD is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2 Surgery

Guinea pigs were anesthetized with isoflurane and stereotaxically implanted with stainless steel cortical screw electrodes and temperature transponders (Bio Medic Data Systems, Seaford, DE) using standard rodent aseptic surgical techniques previously described (McDonough et al., 1995; Shih and McDonough, 1999; see Roberson et al., 2004 for EEG and temperature data). The animals were allowed to recover for at least 5 days prior to experimentation.

2.3 Nerve Agent

Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). The agent was diluted in sterile saline (0.9% NaCl, USP) in concentrations to deliver volumes equal to 0.5 ml/kg and maintained on ice. The most recently determined LD_{50} of 42μg/kg in guinea pig (Hulet et al., 2002) was used in this study.

2.4 Experimental protocol

Animals (N = 150) were randomly assigned to saline (0.5 ml/kg, s.c.) or to one of two sarin doses, 8.4 μg/kg (0.2 x LD_{50}) or 16.8 μg/kg (0.4 x LD_{50}) (s.c.; LD_{50} = 42 μg/kg), and to one of five time points (the day of the tenth exposure and 3, 10, 30 and 100 days post- exposure; time points designated E10, P3, P10, P30 and P100) for a total of 15 groups. The 10 exposures took place Monday-Friday over a two-week period and were designated E1-E10. Each of the 15 groups consisted of 10 animals, with 5 animals assigned to neuropathological assessment and 5 animals assigned to neurochemical assessment. Animals were euthanized at the five time points

described above, and brains were prepared according to the post-sacrifice assessment assignment.

2.5 Cortical dissection and preparation for neurochemical assays

For neurochemical assays, animals were transcardially perfused with saline; the brains were rapidly removed and dissected on ice. Brains were dissected into 6 regions; each region was separated into left and right halves and the halved sections were placed in microfuge tubes, weighed and frozen on dry ice. Our companion article (Roberson et al., 2004) reported the results of brain AChE assays from each of the 6 regions; however, receptor binding was performed only in cortex. During cortical preparation, tissue was homogenized (1:20 w/v) in 50 mM sodium phosphate buffer (PB: pH 7.4). Homogenates were centrifuged at 22, 000 x *g* for 25 min at 4° C, and the supernatant was discarded. The membrane pellet was reconstituted in PB (1:10 w/v) and a sample taken for analysis of protein content by the method of Bradford (1976; kit/Bio-Rad Laboratories, Hercules, CA). One ml of the reconstituted membrane fraction was pipetted into each of several microfuge tubes and centrifuged at 10, 000 x g for 5 min at 4˚ C and the supernatant discarded. The resulting membrane pellets were frozen at -70° for subsequent receptor binding assay or Western blotting.

2.6 Determination of receptor density (Bmax) and affinity (Kd)

Muscarinic m_1AChR cholinergic receptors were assessed in cortex with saturation studies utilizing [^{3}H]-Pirenzepine (Perkin Elmer/NEN, Boston, MA). The cortical membrane pellets (see *2.5*) were reconstituted in 50 mM PB; approximately 150 µg of protein was loaded into each binding tube, and membrane preparation was then incubated at room temperature for approximately 75 min with one of 6 concentrations of radiolabeled ligand (1 to 25nM). Nonspecific binding was assessed in the presence of 10 mM atropine. The incubation was terminated by the addition of 3 ml of ice-cold 50 mM PB (pH 7.4) and rapid filtration through Whatman GF/B filters in a cell harvester (Brandel, Inc., Gaithersburg, MD), followed by two 3 ml washes with ice-cold buffer. The radioligand retained in the filter paper was counted in a liquid scintillation counter with 4 ml of scintillation cocktail (Bio-Safe II, Research Products International, Mt. Prospect, IL). Radioactive count information (samples already normalized for protein content) was submitted to a curve-fitting program (Prism, GraphPad Software, San Diego, CA) designed for the determination of receptor number (B_{max}) and receptor affinity (K_d).

2.7 Western blotting

APP is a trans-membrane domain protein; therefore aliquots of membrane prepared for the receptor binding assay were used for gel electrophoresis and Western blotting using a monoclonal antibody to APP, MAB-348 (Chemicon, Hercules, CA). The pelleted membranes were reconstituted in 50 mM PB and mixed 1:2 with Laemlli buffer, and 20 µl of the Laemlli tissue sample was loaded into each well of a ready-cast 4-12% gradient polyacrylamide gel (electrophoresis reagents, ready-gels, blotting membrane and equipment from Bio-Rad Laboratories, Hercules, CA or Invitrogen Life Technologies, Carlsbad, CA). Following gel electrophoresis, the protein was transferred onto polyvinylidene difluoride (PVDF) blotting membrane and soaked in blocking buffer (10 ml of Tris Buffered Saline withTween-20 (TBST) containing 0.5g non-fat dry milk (Jerrell)) for one hr while being gently swirled in a container on a Belly Button® shaker (Stovall Life Sciences, Inc., Greensboro, NC). The primary antibody and blotting membranes were incubated for 2 hrs and the secondary antibody/membrane for 1 hr.

Following incubation, the PVDF membrane was rinsed in TBST (3 x 10 min), and then treated with a chemiluminescent substrate (AP-BCIP; Sigma, St. Louis, MO) and imaged/quantified using the Kodak Image Station (Eastman Kodak Company, Rochester, NY).

2.8 Neuropathology evaluation for H&E staining

The brain regions selected for detailed light microscopic evaluation of H&E staining were cerebral cortex, piriform cortex, amygdala, hippocampus, caudate/putamen, and thalamus. Brains were blocked in the coronal plane through the center of the infundibulum to ~2 mm caudal, the blocks were placed in imbedding cassettes with the rostral side down, and paraffin-embedded sections were cut at 5 μm thick and stained with H&E. This procedure allows near-identical sections to be evaluated in all test subjects. Heart pathology was analyzed as previously published (McDonough et al., 1995). For the heart tissue, a cross section was prepared that included both ventricular walls and septum. Paraffin-embedded sections of heart were cut 1-10 μm thick and stained with H&E. The same veterinary pathologist, who was unaware of the experimental history of a given subject, evaluated all H&E-stained tissues from all subjects in this study as previously described (McDonough et al., 1995).

2.8 Neuropathology evaluation for MAP-2 staining

Since H&E staining would not allow detection of any subtle signs of neuronal injury that might appear following low-level exposure, MAP-2 staining was performed using the avidin-biotin-peroxidase complex (ABC) method described by Hsu et al. (1981). Currently, no atlas of the guinea pig brain exists; consequently, a rat atlas (Paxinos and Watson, 1998) was used as a guide to approximate the brain regions selected for MAP-2 evaluation. The regions selected were between Bregma -5.30 mm and -6.04 mm, and included piriform and entorhinal cortices, the hippocampal formation and the amygdala. Paraffin sections dewaxed in xylene and hydrated in graded alcohol were subjected to antigen retrieval procedures using a microwave oven. Following two rinses in phosphate buffered saline (PBS), sections were incubated in 5% normal serum for 30 min to suppress endogenous tissue immunoglobulin that could react with the biotinylated secondary antibody and then sequentially incubated in MAP-2 primary antibody (MAb clone AP18; 1:100 dilution; Lab Vision, Fremont, CA)) for 18 hours; biotinylated secondary antibody for one hr; and ABC solution for 30 min. Immunoreaction product was developed in a mixture of diaminobenzidine tetrahydrochloride (DAB) and urea hydrogen peroxide (Sigma, St. Louis, MO).

2.9 Data analysis

Two-way (dose, days) analysis of variance (ANOVA) was used to determine the effect of experimental manipulations of the five time points: day of exposure 10 (E10) and 3 (P3), 10 (P10), 30 (P30) and 100 (P100) days post-exposure. If overall main effects were significant, all possible comparisons were made using the Neuman-Keuls test. Statistical significance was defined as $P<0.05$.

III. RESULTS

3.1 Receptor binding using M_1AChR ligand [3H]-Pirenzepine

A two-way ANOVA was performed (group/day) to assess M_1AChR density (B_{max}), (Figure 1); there was no effect of sarin treatment ($P=0.5$), and there was no effect of treatment day

($F_{4,66}$=0.68; P=0.6) and no group x day interaction (P=0.7). Likewise, a two-way ANOVA was performed to assess M_1AChR affinity ($K_{d;}$ dissociation constant, an indicator of receptor affinity) (Figure 2), and no effect of sarin treatment was observed (P=0.6). Additionally, there was no effect of treatment day (P=0.1) and no group x day interaction (P=0.2).

3.2 Western blotting using antibody to APP

APP levels from the 3 groups (saline and 0.2 and 0.4 x LD_{50} sarin) were compared within each of the five time points examined, E10, P3, P10, P30 and P100. There was no significant effect of sarin treatment on cortical levels of membrane-bound APP (Figure 3, Panels A-E) at E10 (P=0.3), P10 (P=0.9) and P100 (P=0.5). There was, however, a small but significant increase in band intensity in both the 0.2 and 0.4 x LD_{50} groups at P3 (P=0.03), but an increase in only the 0.4 x LD_{50} group at P30 (P=0.03).

3.3 Neuropathological evaluation of H&E and MAP-2 staining

When brain slices that had been stained with H&E were evaluated, no brain lesions were observed in any brain region at any time point. Further, evaluation of H&E staining did not reveal any cardiac abnormalities in any group or time point. MAP-2 staining, capable of detecting neuronal damage or injury, was conducted in a subset of animals (100-day time point only), in brain sections containing the piriform and entorhinal cortices, the hippocampal formation and the amygdala. Control (saline) animals exhibited typical patterns of MAP-2 immunoreactivity (Figure 4, Panel A/hippocampus), and the evaluation revealed no change in immunoreactivity in the sarin animals in any brain region examined as would be expected with some type of neuronal injury (Figure 4, Panels B-D/hippocampus). Due to technical difficulties, we were unable to evaluate MAP-2 staining at the remaining time points.

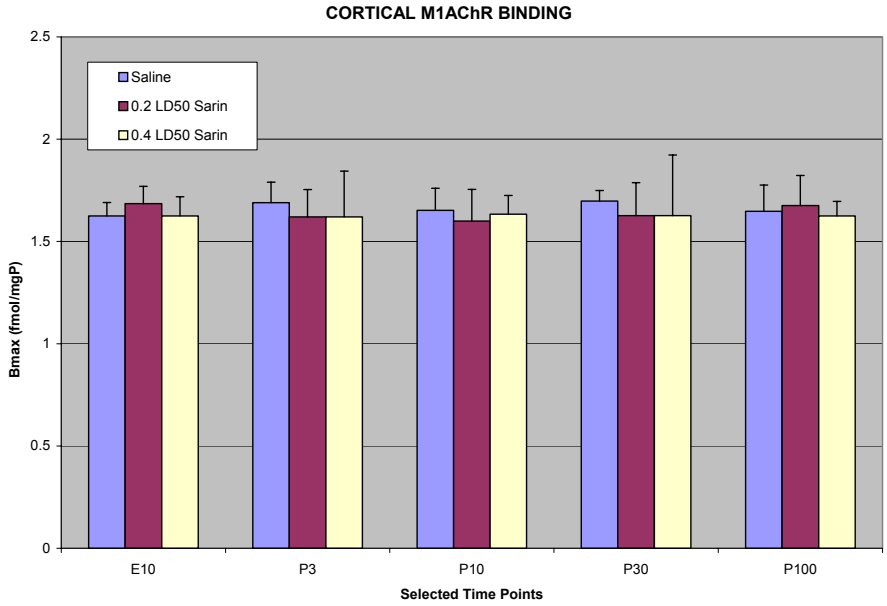

Figure 1: Effect of Low-Level Sarin Exposure on Cortical M1AChR Density: There was no effect of sarin treatment (*P*=0.5) on receptor density (B_{max}) at any of the five time points examined, E10, P3, P10, P30 or P100, when sarin animals were compared with saline animals. (N = 5/group at each time point.)

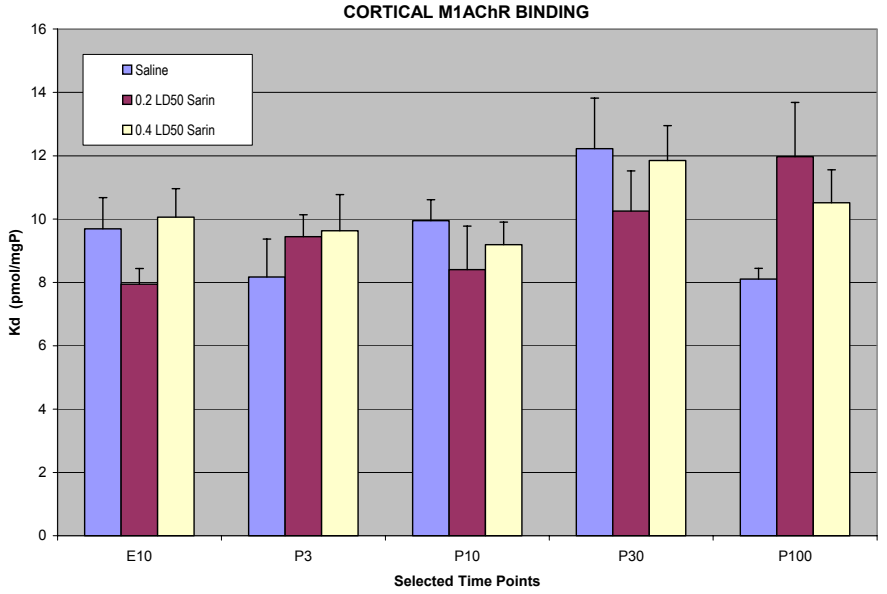

Figure 2: Effect of Low-Level Sarin Exposure on Cortical M1AChR Affinity: There was no effect of sarin treatment (*P*=0.6) on receptor affinity (dissociation constant, K_d) at any of the five time points examined, E10, P3, P10, P30 or P100, when sarin animals were compared with saline animals. (N = 5/group at each time point.)

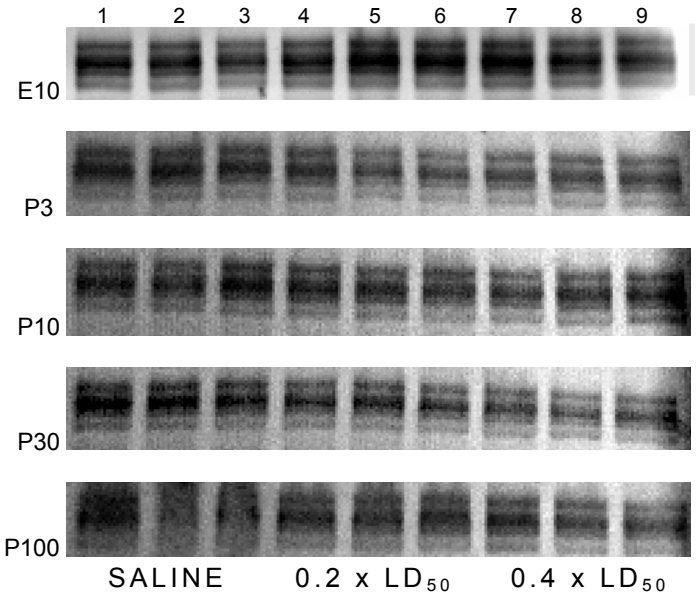

Figure 3: Effect of Low-Level Sarin Exposure on Levels of Cortical APP: There was no effect of sarin treatment on cortical APP_m at E10 (*P*=0.3), P10 (*P*=0.) and P100 (*P*=0.5). There was a significant increase in band intensity in the 0.2 and 0.4 x LD_{50} groups at P3 (*P*=0.03) and an increase in only the 0.4 x LD_{50} group at P30 (*P*=0.03). (Bands1-3: saline; 4-6: 0.2 x LD_{50} sarin; 7-9: 0.4 x LD_{50} sarin; N = 5/group at each time point.) Apparent are the three main isoforms of the protein, APP_{695}, APP_{751} and APP_{770} (approximately 120-130 kDa).

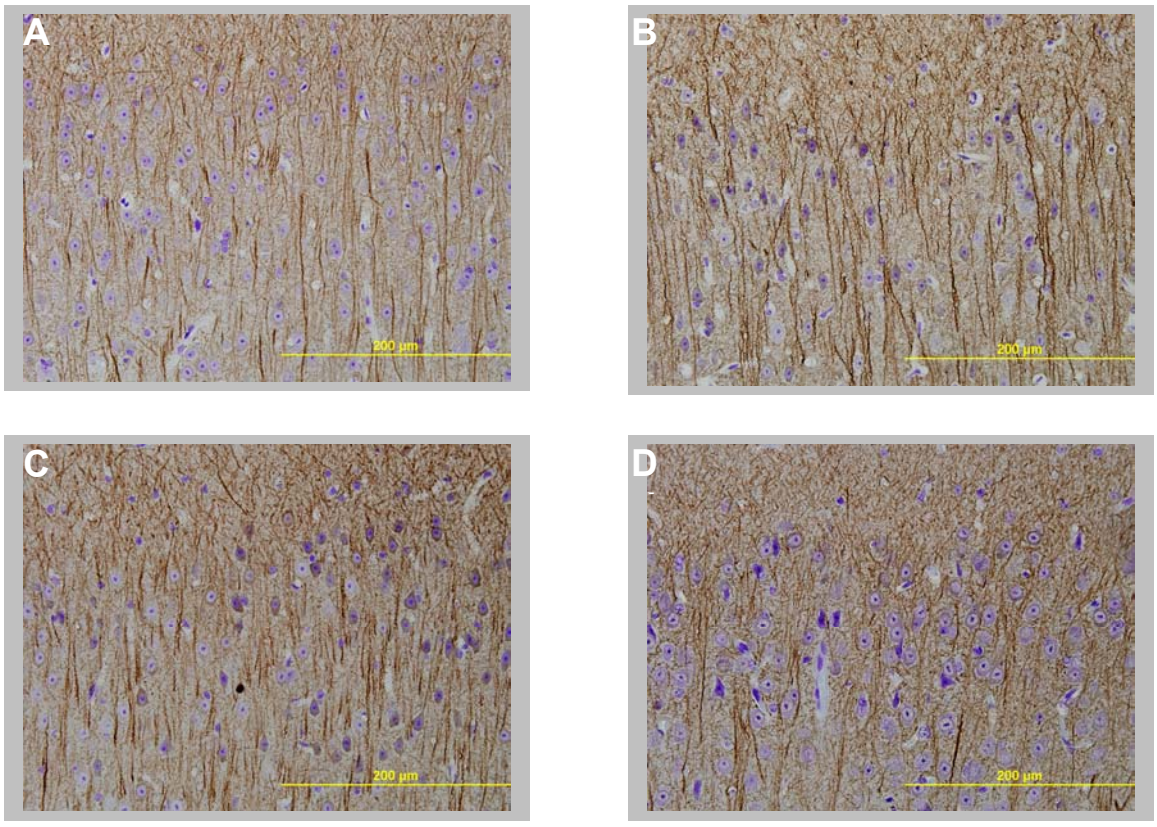

Figure 4: Effect of Low-Level Sarin Exposure on MAP-2 Staining in the Hippocampus: MAP-2 staining was evaluated in brain slices from animals of 4 treatment groups at the P100 time point. Staining was observed in hippocampal dendrites and faintly in nerve cell bodies (Panel A: saline; Panel B: 0.2 x LD_{50} sarin; Panel C: 0.4 x LD_{50} sarin; Panel D: 0.5 x LD_{50} sarin). Brain sections of control animals (Panel A) showed typical patterns of MAP-2 immunoreactivity, and no change in immunoreactivity was observed in sarin-exposed animals (Panels B-D) when compared with controls. (40 X objective.)

IV. DISCUSSION

We previously reported that repeated exposure to either 0.2 or 0.4 x LD_{50} sarin (8.4 or 16.8 μg/kg) does not result in visible signs of nerve agent intoxication or changes in body temperature or body weight, in spite of significant reductions in brain (0.4 x LD_{50}) and RBC AChE activity (0.2 and 0.4 x LD_{50}) following E10 (Roberson et al., 2004). Increases were observed in both electroencephalographic (EEG) $beta_2$ power (during the exposure period) and in flinch threshold (following the 10-day exposure period). However, no differences were observed among groups on those parameters at 100 days post-exposure, the last time point examined, and both brain and RBC AChE activity had returned to baseline levels by 100 days as well. (For discussion, see Roberson et al., 2004.)

Even though it has been reported that low levels of OP exposure result in a decrease of muscarinic receptor binding (Churchill et al., 1984-a, 1985; Russell et al., 1986; McDonald et al., 1988; Raffaele et al., 1990; Fitzgerald and Costa, 1992; Katz and Marquis, 1992; Ehrich et al., 1994; Khan et al., 2000; Zheng et al., 2000), the cortical m_1AChR receptor binding assay ([^{3}H]-Pirenzepine) conducted in this study did not reveal any differences among groups in either receptor density or receptor affinity. No change in receptors was expected in the 0.2 x LD_{50} sarin group since brain AChE activity in this group remained at control levels across the five time points sampled (Roberson et al., 2004). This was true in all 6 brain regions assayed, including the cortex, the brain region chosen for receptor binding in this study.

As noted previously (Roberson et al., 2004), the possible variations of experimental factors used in sub-toxic nerve agent studies--such as the species, nerve agent, route of administration and in the case of receptor binding, receptor ligand and autoradiography vs. filtration assay--underscore the difficulty of reconciling data to form a coherent hypothesis. It should be further noted that while Churchill et al. (1984-a) designate their chosen doses of soman as "sublethal," the death rate for their exposure regimen was 38%, adding to the difficulty of comparing their receptor binding results with those of this asymptomatic exposure study. Another previous study found reduced muscarinic binding sites in calf caudate at PX concentrations that did not significantly inhibit AChE activity (Katz and Marquis, 1989); however, the ligand used was not specific for the M_1 subtype but was a non-specific muscarinic ligand. Khan et al. (2000) found changes in both cortical nicotinic (nAChR) and muscarinic binding in rats at concentrations of sarin (0.01 and 0.1 x LD_{50}) that were clearly below the doses used in the current study; however, the muscarinic ligand was to the M_2 subtype, the longest time point sampled was 20 hrs and the injection route was intramuscular (i.m.) and not s.c. as in the current study. The most notable difference, however, is that Khan et al. based the sarin LD_{50} on 100 μg/kg rather than on the lower 42 μg/kg of this study.

Much has been learned about the regulation of APP metabolism in both normal and disease states (reviewed in Roberson and Harrell, 1997). It has been noted that "APP processing in those neurons receiving cholinergic input is driven by cholinergic stimulation, presumably by the activation of m_1AChR on cholinoceptive target cells" (Slack et al., 1995). Although other neurotransmitter systems participate in APP processing (Nitsch et al., 1992, 1996; Lee et al., 1995; Muller et al., 1997), experiments with transfected cells have demonstrated that m_1/m_3AChRs are positively coupled to APP secretion (Oltersdorf et al., 1989). Furthermore, Rossner et al. (1997) reported that APP secretion was strongly positively correlated to the density of m_1AChR. In the current study, there was a transient increase in APP band intensity in both agent groups at P3, but only the 0.4 x LD_{50} group showed an increase by P30 and all groups

were equivalent by P100. Thus, the current study failed to find long-lasting sarin-related differences in cortical levels of membrane-bound APP. This is in agreement with one study that used immunochemical staining with a monoclonal antibody to APP on brain slices from guinea pigs subjected to the same exposure regimen as the current study, but using 0.4 x LD_{50} VX (Kan et al., 2002). Following the exposure period, an increase in APP staining was observed in the hippocampal CA1 region; however, a return to normal levels of APP was seen two weeks later. Since no effect of nerve agent treatment was found in the density or affinity of cortical M_1 receptors, the transient increase seen in this study could have been mediated by a transient sarin-related increase in APP mRNA rather than by localized receptor/synaptic changes. As noted previously, Blanton et al. (2004) observed an increase in APP mRNA in mice following repeated exposure to low levels of the nerve agent VX, although all up-regulated gene expression had returned to pre-exposure levels by two weeks post-exposure in that case.

The results of the H&E staining in brain and heart confirmed that, as expected, there were no lesions in heart or in any brain region examined. A 1998 study by McDonough et al. reported that neural lesions detected following soman-induced seizures in the rat are correlated with increases in delta band activity. This is in line with the analysis of electroencephalographic activity from the animals of the current study that demonstrated an exposure period increase in beta $_2$--and not delta--band activity (Roberson et al., 2004). Additionally, the beta $_2$ increase did not persist past P3. A study using different staining techniques but the same exposure regimen with a 0.4 x LD_{50} dose found no evidence of cytopathology or axonopathy in the medulla, pons, cerebellum and midbrain (Petras et al., 2004). The following pathological observations were present only in a select number of animals receiving the 0.4 x LD_{50} dose: the *stria terminalis thalami* and the *habenular nuclei* were affected bilaterally (most thalamic regions were not affected), and limited fiber degeneration was seen in the cingulate cortex. In light of these pathological changes, albeit limited at the 0.4 x LD_{50} dose, it should be noted that the nerve agent was different (soman; 0.4 x LD50 = 122 μg/kg) and neurological signs were present at the time of exposure, even at the 0.2 x LD_{50} dose. This is in contrast with the lack of neurological signs observed in the current study, suggesting a more potent central effect in the soman study.

While neuronal death in the form of visible lesions was not expected in the current study, the possibility of subtle neuronal injury was assessed using MAP-2 staining. MAP-2 is an important part of the cytoskeleton, and can be visualized in nerve cell bodies and dendrites but not in axons (Matus, 1994). MAP-2 staining has also been demonstrated to be a marker of seizure-related brain damage following acute soman administration; Ballough et al. (1995) found reductions in MAP-2 immunoreactivity following soman-elicited seizures in rats. In the current low-level study, however, there was no change in MAP-2 immunoreactivity when the 0.2 and 0.4 x LD_{50} sarin animals were compared with controls. It should be noted that only the P100 time point was examined; however, an additional 0.5 x LD_{50} sarin group was included and no change in MAP-2 immunoreactivity was observed in the higher dose at P100. These results suggest that no persistent neuronal injury resulted from this regimen of low-level sarin exposure.

The results of our previous study (Roberson et al., 2004) found no permanent or persistent effect of sarin on any parameter examined including physical, behavioral, electroencephalographic or biochemical. This, taken together with the current neurochemical and neuropathological data, suggests that the initial depression of AChE is not of sufficient magnitude and duration to result in persistent changes that cause long-lasting neurochemical or neuropathological alterations.

V. REFERENCES

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VI. ABBREVIATIONS

(ABC) avidin-biotin-peroxidase complex

(ACh) acetylcholine

(AChE) acetylcholinesterase

(AD) Alzheimer's disease

(ANOVA) analysis of variance

(APP) amyloid precursor protein

(B_{max}) receptor number or density

(DAB) diaminobenzidine tetrahydrochloride

(DFP) diisopropyl fluorophosphate

(EEG) electroencephalographic

(H&E) hematoxylin and eosin

(K_d) receptor affinity

(mAChRs) muscarinic receptors

(MAP-2) microtubule-associated protein

(OP) organophosphorus

(PB) sodium phosphate buffer

(PNS) phosphate buffered saline

(PVDF) polyvinylidene difluoride

(PX) paraoxon

(QNB) quinuclidinyl benzilate

(RBC) red blood cell

(TBST) Tris buffered saline withTween-20